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Nox4 contributes to the hypoxia-mediated regulation of actin cytoskeleton in cerebrovascular smooth muscle

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Abstract

Ischemia/reperfusion and the resulting oxidative/nitrative stress impair cerebral myogenic tone via actin depolymerization. While it is known that NADPH oxidase (Nox) family is a major source of vascular oxidative stress; the extent and mechanisms by which Nox activation contributes to actin depolymerization, and equally important, the relative role of Nox isoforms in this response is not clear.

Aim—To determine the role of Nox4 in hypoxia-mediated actin depolymerization and myogenic-tone impairment in cerebral vascular smooth muscle.

Main methods—Control and Nox4 deficient (siRNA knock-down) human brain vascular smooth muscle cells (HBVSMC) were exposed to 30-min hypoxia/45-min reoxygenation. Nox2, Nox4, inducible and neuronal nitric oxide synthase (iNOS and nNOS) and nitrotyrosine levels as well as F/G actin were determined. Myogenic-tone was measured using pressurized arteriography in

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Disclosures

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Author Contributions

Participated in research design: Ergul, Coucha

Performed experiments: Coucha, Abdelsaid, Li

Performed data analysis: Coucha, Johnson, Ergul

Contributed new reagents or analytic tools: Laszlo

Wrote or contributed to the writing of the manuscript: Coucha, Abdelsaid, Li, Johnson, Laszlo, El-Remessy, Fagan and Ergul

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middle cerebral artery isolated from rats subjected to sham, 30-min ischemia/45-min reperfusion or ex-vivo oxygen glucose deprivation in the presence and absence of Nox inhibitors.

Results—Nox4 and iNOS expression were significantly upregulated following hypoxia or ischemia/reperfusion. Hypoxia augmented nitrotyrosine levels while reducing F actin. These effects were nullified by inhibiting nitration with epicatechin or pharmacological or molecular inhibition of Nox4. Ischemia/reperfusion impaired myogenic-tone, which was restored by the selective inhibition of Nox4.

Conclusion—Nox4 activation in VSMCs contributes to actin depolymerization after hypoxia, which could be the underlying mechanism for myogenic-tone impairment following ischemia/reperfusion.

Introduction

The brain is a unique organ of high metabolic demand, which requires tight regulation of blood flow and oxygen delivery for survival. Myogenic response is one of the main regulatory paradigms of cerebral blood flow. It describes the intrinsic ability of vascular smooth muscle cells (VSMC) to vasoconstrict or vasodilate according to changes in pressure maintaining constant blood flow [1, 2]. Therefore, a well-developed myogenic tone is critical for achieving optimum vascular resistance under increased cerebral perfusion pressure. The development of myogenic response in vascular smooth muscle requires the interplay of various complex mechanisms to achieve a sequence of events: 1) detection of wall strain/stretch which leads to membrane depolarization, 2) calcium entry, protein phosphorylation and constriction, 3) activation of the Rho-associated kinase and protein kinase C (PKC) pathways inducing calcium sensitization, and 4) actin polymerization and force transmission enhancement [3–5]. Actin polymerization is significantly disturbed after ischemia/reperfusion (I/R) injury. The filamentous (F): globular (G) actin ratio is particularly susceptible to oxidative stress. We and others have shown that peroxynitrite mediates a decrease in F actin of VSM after periods of oxygen deprivation leading to loss of tone [6, 7]. However, the exact mechanism by which I/R injury induces actin depolymerization needs to be determined.

Early restoration of cerebral blood flow is the goal for treatment of ischemic stroke. However, it paradoxically enhances reactive oxygen species (ROS) generation, which aggravates tissue damage [8–10]. Therefore, targeting ROS is a logical strategy in stroke management. Nevertheless, preclinical studies and clinical trials of antioxidant therapies have failed repeatedly. One potential reason was thought to be the untargeted use of antioxidants [11, 12]. Therefore, identifying the potential source of ROS after I/R may provide a better therapeutic approach for stroke management.

Our previous studies demonstrated a link between excess peroxynitrite generation, actin nitration and loss of myogenic tone [7, 13]. Here, we attempted to identify the source of peroxynitrite parent radicals after short periods of oxygen glucose deprivation. Peroxynitrite is a strong oxidant, which is produced from the reaction of superoxide anion and nitric oxide free radicals. Nitric oxide exerts both neuroprotective and neurotoxic effect in stroke, which counts on the nitric oxide synthase (NOS) from which it is generated. Experimental studies

demonstrated the deleterious effect of both neuronal (nNOS) and inducible (iNOS) in cerebral ischemic injury [14]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a major source of vascular oxidative stress. It catalyzes the transfer of electrons through flavin adenine dinucleotide from NADPH to molecular oxygen to produce superoxide anion. The family of NADPH oxidase comprises of seven isoforms, annotated as Nox1-5 and DUOX1-2, which exhibit unique patterns of tissue specificity [15–17]. Nox4 and Nox2 are highly expressed in the cerebral vasculature compared to systemic arteries [18–20], making both isoforms potential sources of ROS after stroke. Nox2 or Nox4 knock-out in different animal models showed impressive neuroprotection after I/R injury, suggesting that both isoforms are potential targets for stroke management [21–23]. Our goal was to investigate the contribution of Nox2 and Nox4 to nitration-mediated loss of myogenic tone and regulation of actin dynamics. We specifically tested the hypothesis that hypoxia-induced upregulation of Nox4 expression contributes to increased peroxynitrite formation and nitration leading to actin depolymerization.

Materials and Methods

Animals

Male Wistar rats (250–350 gm) (Harlan; Indianapolis, ID) were used in all experiments. The animals were housed at Augusta University animal care facility, which is approved by the American Association for Accreditation of Laboratory Animal Care. Institutional Animal Care and Use Committee (IACUC) approved the protocol of this study. Animals were fed standard rat chow and tap water ad libitum. All animals were anesthetized with pentobarbital sodium (Fatal-Plus, Vortech Pharmaceuticals Ltd; Dearborn, MI) then sacrificed by decapitation.

Models of hypoxia/ischemia and drug treatment

***In vitro* cell culture model**—Primary cultures of HBVSMC and supplies were purchased from Cellscience (Carlsbad, CA). Cells (passages 3–6) were switched to overnight serum free media prior to exposure to 30 min hypoxia (<0.2% O₂, 95% N₂ and 5% CO₂) and 45 min reoxygenation. The selective nitration inhibitor (–)-epicatechin (Epi) (150 μM; Sigma Aldrich; St. Louis, MO) [24] or the selective iNOS inhibitor, N-[[3-(aminomethyl)-phenyl]methyl]-ethanimidamide dihydrochloride (1400W) (10 μM; Sigma Aldrich; St. Louis, MO) [25] was added during reoxygenation.

***Ex vivo* model**—To determine the contribution of Nox4 on myogenic tone, naïve middle cerebral arteries (MCAs) were pressurized and exposed to oxygen-glucose deprivation (OGD, 95% N₂/5% CO₂, glucose 1 mM) for 30 min followed by 20 min reoxygenation. Relatively specific small-molecule Nox4 inhibitor, VCC588646 (2-[N'-(3,4-Dihydroxy-benzylidene)-hydrazino]-N-(3-nitro-phenyl)-2-oxo-acetamide (100 μM, Vichem-Chemie, LTD) was added during reoxygenation [26, 27]. Pressure-diameter curves were obtained across pressure ranges.

***In vivo* model**—Focal cerebral ischemia was achieved using the monofilament suture middle cerebral artery occlusion (MCAO) model [28, 29]. Rats were subjected to sham

surgery or 30 min of MCAO/45 min reperfusion. Blood flow was measured by a scanning laser Doppler (Perimed; North Royalton, OH) to assess MCAO and reperfusion. The common carotid was located in the sham-operated group, but animals were not subjected to MCAO and were sacrificed as the experimental group. Apocynin, an antioxidant and NADPH oxidase inhibitor, was administered as a single dose at reperfusion (50 mg/kg i.p.) [30].

Pressurized arteriography system

MCA segments from ischemic hemisphere were cannulated and pressurized in an arteriograph chamber as previously showed by our group [31]. To determine the myogenic tone, pressure-diameter curves were obtained, first in the presence of Ca^{2+} (active diameter), and then in Ca^{2+} -free buffer (passive diameter) with the addition of 0.2 mM papaverine hydrochloride. Myogenic Tone % = $1 - (\text{Active diameter/Passive diameter}) \times 100$.

Western blot analysis

Nox4 and Nox2 expression in isolated MCAs and HBVSMC were analyzed by western blot. Snap frozen MCAs were homogenized and equal volumes of cell lysate and homogenized tissue were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Nox4 and Nox2 were determined by using anti-Nox4 antibody (1:500, ab109225) and anti-Nox2 antibody (1:500, ab31092), respectively (Abcam; Cambridge, Ma). Nox1, nNOS and iNOS expression were determined using anti-Nox1 antibody (1:250 Abcam; Cambridge, Ma, ab55831), anti-nNOS antibody (1:250) and anti-iNOS antibody (1:250), respectively (Santa Cruz Biotechnology; Dallas, TX) in HBVSMC. Primary antibodies were detected using horseradish peroxidase-conjugated antibody and enhanced chemiluminescence. Blots were imaged and band intensity was quantified by the densitometry software (Alpha Innotech; Santa Clara, CA).

Differential ultracentrifugation of homogenates for measurement of filamentous- and globular-actin

HBVSMC were harvested by scraping thoroughly with cell scraper after the addition of prewarmed (37°C) lysis buffer as suggested by the manufacturer (Cytoskeleton, Denver, CO). Cell lysates were quickly homogenized using a 200 µl pipette tip and placed on ice until all samples are completed, and then incubated at 37°C for 10 min. Cell lysates were transferred to a prewarmed (37°C) ultracentrifuge and spun at 100,000 g for 1 h at 37°C to separate the G-actin (supernatant) and F-actin fractions (Beckman, Fullerton, CA). F-actin depolymerization buffer was added to each pellet and incubated on ice for 1 hr to allow actin depolymerization to occur. Equal volumes of loading buffer were added to each of the supernatant and the pellet, and then boiled for 5 min. Actin quantification was performed by SDS-PAGE and western blot analysis.

F-actin and nitrotyrosine staining and quantification

HBVSMC were pressure fixed in methanol-free 4% paraformaldehyde in PBS for 10 min. Fixed cells were then stained for F actin with Oregon Green 488-conjugated phalloidin fluorescent probe (Invitrogen, Carlsbad, CA), and for nitrotyrosine with a mouse anti-

nitrotyrosine primary antibody at 1:50 and a goat anti-mouse IgG conjugated to Cy5 (Molecular Probes) secondary antibody at 1:1000. Z-stacks (2.1 μm ; 0.3 μm intervals) of the vascular smooth muscle were captured at 40X using an LSM 510 upright confocal microscope and processed using Zen 2008 software. ImageJ software (National Institutes of Health, Bethesda, MD) was used to quantify F actin and nitrotyrosine volume intensity.

Tissue markers of nitrosative stress

Total nitrotyrosine levels were determined in cell lysate via slot blot analysis as described previously [7]. Equal amounts of cell lysate were immobilized onto nitrocellulose membrane. Relative levels of nitrotyrosine were detected by an anti-nitrotyrosine monoclonal antibody (Millipore; Lake Placid, NY, 05-233) and were quantified by the densitometry software (Alpha Innotech; Santa Clara, CA).

Silencing Nox4 expression

Transfection of HBVSMC was performed using Amaxa nucleofector and a kit for primary SMC according to the manufacturer's protocol (Lonza; Allendale, NJ). Optimization experiments showed that A-033 program and 300nM of Nox4 siRNA (OriGene Technologies, Rockville, MD) gave the maximum transfection efficacy for HBVSMC. Cells were transfected with siRNA and pmaxGFP then were left in complete medium for 24 hours to recover. Experiments were performed within 72 hours after transfection.

Dihydroethidium (DHE) staining assay for superoxide formation

Superoxide anion levels were quantified by fluorometric assay of dihydroethidium (DHE) (Life Technologies, Grand Island, NY), which intercalates within the cell's DNA and stains its nucleus a bright fluorescent red when oxidized (excitation/emission 518/606). The culture media was replaced with phosphate buffered saline containing 10 μM DHE [32] and then cells were exposed to 30 min hypoxia/45 min reoxygenation. Fluorescence intensity was immediately quantified at the end of the reoxygenation by high performance multimode plate reader (Biotek, Winooski, VT).

Quantitative real time PCR

A one-step quantitative RT-PCR kit (Invitrogen) was used according to manufacturer's instructions to amplify 10 ng HBVSMC mRNA. PCR primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Nox4 sequence (5'-GCAGGATCCGTCATAAGTCATCCCTCAGA-3 forward, 5'-GCTGTTAACGTCGACTCAGCTGAAAGACTCTTTAT-3 reverse). Actin sequence (5'-CCTGGCACCCAGCACAAT-3 forward, 5'-GCTGATCCACATCTGCTGGAA-3 reverse). Quantitative PCR was performed using qPCR system (Applied Biosystems, Life Technologies). The expression of Nox4 was normalized to actin.

Statistical analysis

All the data are expressed as mean $\pm$ SEM, statistical analyses were performed using SAS[®] 9.3 (SAS Institute Inc., Cary, NC). A rank transformation was used prior to analysis where needed to stabilize variance across groups. For arteriography studies, area under the curve

(AUC) across the pressure range (40–180 mmHg) was calculated. A series of 2 Stroke (no vs. yes) X 2 treatment (TRT) (no vs. yes) ANOVAs with interaction were used to determine the effect of apocynin on myogenic tone. Differences between sham vs. I/R injury and normoxia vs. hypoxia conditions were tested using two-sample t-tests. A 2 Oxygen (normoxia vs. hypoxia) by 2 TRT (no vs. yes) ANOVA was used to test for effects due to epicatechin or Nox4 siRNA and their interaction with oxygen level. A significant interaction would indicate a differential effect of the TRT due to the level of oxygen present. In the absence of a significant interaction, the main effects are considered to be additive. A Bonferroni post-hoc multiple comparison test was used for significant interactions. Significance for all tests was determined at $\alpha=0.05$.

Results

Hypoxia induced actin depolymerization in HBVSMC

Immunofluorescent staining and western blot analysis revealed a shift in F:G actin ratio towards increased G formation and reduced F actin levels after 30 min hypoxia/45 min reoxygenation in HBVSMC (Fig. 1A,B). Nitrotyrosine was measured as a marker of peroxynitrite generation. Hypoxia augmented nitrotyrosine level (Fig. 1C,D), which was associated with reduced F actin (Fig. 1D). Treating the cells at reoxygenation with epicatechin, a selective nitration inhibitor that has no effect on oxidation, restored nitrotyrosine and F actin levels back to normal (Fig. 1C,D) in the hypoxic group with no effect in the normoxic group indicating a disease by treatment interaction ($p<0.001$). To unravel the sources of peroxynitrite parent radicals, the expression of various Nox and NOS isoforms were determined. Nox4 and iNOS expressions were upregulated compared to normoxia (Fig. 2A,D) but there was no change in Nox2, Nox1 and nNOS (Fig. 2B,C,E).

Nox4 knock-down mitigates hypoxia-induced actin depolymerization

Nox4 knockdown nullified the increase of Nox4 expression after hypoxia (Fig. 3A,B). Since the interaction between NADPH oxidase homologues is essential to be considered, we determined the impact of Nox4 knockdown on the expression of Nox1 and Nox2. The expression of both isoforms was not altered by silencing of Nox4 (Fig. 3C,D). Furthermore, superoxide and nitrotyrosine levels were significantly increased after hypoxia and Nox4 silencing nullified this effect in HBVSMC exposed to 30 min hypoxia/45 min reoxygenation (Fig. 4A–C).

The role of iNOS in peroxynitrite generation was confirmed by the use of iNOS inhibitor which blunted the increase in nitrotyrosine levels after hypoxia (Fig. 4D). Finally, by measuring F actin levels via immunoblotting and immunostaining we showed that Nox4 knockdown restored F:G (Fig. 5A) back to normal and blunted the decrease of F actin levels compared to scrambled RNA (Fig. 5B).

Effect of NADPH oxidase inhibition on vascular reactivity after I/R injury

To determine the functional relevance in vessels, we measured Nox protein levels in MCAs after I/R. We found that Nox4 expression was significantly elevated in isolated MCAs after I/R injury compared to sham (Fig. 6A). I/R injury had no effect on Nox2 expression (Fig.

6B) supporting our in-vitro data. Next, myogenic tone was measured in MCAs isolated from animals treated with apocynin, an antioxidant and a broad NADPH oxidase inhibitor, at reperfusion. Analysis of AUC indicated a disease by treatment interaction such that apocynin improved the myogenic response in animals subjected to I/R injury ($p < 0.05$ vs vehicle stroke) and decreased the tone in the sham group ($p < 0.05$ vs sham + Apo) (Fig. 6C). To specifically inhibit Nox4, MCA isolated from control Wistar rats were exposed to ex-vivo OGD followed by reoxygenation with or without a specific Nox4 inhibitor that recently became available. We found that inhibiting Nox4 significantly improved myogenic function (Fig. 6D).

Discussion

Actin polymerization plays a prominent role in the regulation of myogenic tone [3, 4], which is impaired following stroke. It has been previously shown that excess peroxynitrite reduces F actin in vascular smooth muscle via increased nitration leading to loss of tone [6, 7]. Recently we showed that myogenic tone was not affected by I/R injury in superoxide dismutase1 (SOD1) transgenic rats, which emphasize the role of oxidative stress in regulation of myogenic tone [33]. In the current study, we attempted to identify the sources of peroxynitrite parent radicals and the exact mechanisms that contribute to actin depolymerization after hypoxia. We focused on VSMCs since myogenic reactivity is an inherent property of these cells.

Experimental studies in NOS knockout mice demonstrated the protective role of eNOS after cerebral ischemia, while nNOS activation and iNOS expression are shown to be detrimental to ischemic brain [36]. In the current study, we found that the expression of iNOS isoform was significantly elevated after hypoxia, but there was no change in nNOS expression. Moreover, treating HBVSMC with the selective iNOS inhibitor restored nitrotyrosine levels after hypoxia. While further studies using NOS pharmacological inhibitors and NOS deficient animal models would confirm the role of iNOS after ischemic injury, current findings suggest that iNOS could be the source of nitric oxide in peroxynitrite generation in our *in vitro* model of hypoxia.

Nitric oxide is a small hydrophobic molecule that can move freely across the membrane. Therefore, most of the exerted effect of peroxynitrite on the actin cytoskeleton is restricted to the region of superoxide anion generation. Thereby, we were interested in identifying the potential sources of superoxide that lead to peroxynitrite generation after hypoxia within VSMC. Nox proteins are well-established sources for ROS in various vascular diseases including brain injury [15, 16, 20, 37]. Several studies showed that enhanced Nox4 expression was accompanied with an increase not only in hydrogen peroxide but also superoxide [38–42]. Nox4 silencing has been a promising strategy in improving neurovascular outcomes following I/R injury [21–23]. However, the role of Nox4 in myogenic tone impairment after stroke is still unexplored. Nox4 is localized at the focal adhesions [43–46], which are the major sites where transmembrane adhesion receptors link intracellular cytoskeletal structural proteins and signaling molecules [20, 47, 48]. We have previously shown that gp91-tat, a small peptide that inhibits Nox-1, 2 and 4, restored myogenic tone after oxygen glucose deprivation [7]. Therefore, in the current study we built

upon our past studies suggesting an important role for NADPH oxidases in regulation of myogenic tone. We found an increase in only Nox4 isoform expression after hypoxia in HBVSMC, which was associated with an increase in nitration, superoxide level and actin depolymerization. These effects were attenuated by silencing Nox4 in HBVSMC. We also showed that inhibiting nitration with epicatechin restored F actin levels. Epicatechin is a flavonoid and one of the green tea extracts. It selectively blocks the ability of peroxynitrite to nitrate tyrosine residues but has no effect on thiol oxidation [24, 34, 35].

Consistent with *in vitro* studies, Nox4 expression was increased in isolated MCAs after I/R injury, while Nox2 expression showed no change. These results suggest that Nox4, but not Nox2, could be involved at early time points in the pathophysiology of vascular I/R injury of the cerebral vasculature. Ischemic MCAs isolated from rats after receiving apocynin experienced normal myogenic response. A potential limitation of apocynin is that its mechanism of action is still controversial and its antioxidant effects may not be through Nox inhibition [49]. Therefore to overcome this limitation we used a recently described and relatively specific inhibitor of Nox4 [26, 27]. Interestingly, we found that apocynin impaired myogenic response in sham arteries. These findings are in agreement with our previous study, in which we demonstrated a decrease of myogenic tone in sham arteries after scavenging peroxynitrite [7]. Taken together, our results suggest that oxidative signaling is involved in regulation of vascular tone and emphasize the importance of a well-balanced redox state for myogenic function.

Although, ROS are known to impair vascular tone via modifying actin polymerization, one should keep in mind that other players could affect actin cytoskeleton. It has been shown that knocking down p47phox in mice hearts impaired actin polymerization in non-ROS-dependent mechanism [50]. However, since excess ROS generation after I/R injury or hypoxic insult plays a pivotal role in vascular injury [8, 9, 51], we focused on identifying sources of ROS generation and ROS-dependent mechanisms of actin depolymerization. We believe that this understanding could lead to new therapeutic interventions for ischemic stroke. The data from the present study would be strengthened by determining Nox4 and Nox2 expression in endothelium denuded vessels. We could not perform those measurements due to the limited amount of cerebrovascular tissue isolated from our stroked groups. In order to overcome this limitation, we used HBVSMCs in our *in vitro* study to confirm the overexpression of Nox4 after oxygen deprivation. Although cultured VSMCs enabled us to study signaling events, we are cognizant that they differ from native cells. Therefore, further studies using Nox4 pharmacological inhibitors and Nox4 deficient mice are crucial to confirm the role of Nox4 in actin polymerization and myogenic tone after ischemic injury.

Conclusion

Nox4 plays an important role in hypoxic vascular injury. It is upregulated after ischemic stroke and hypoxic insult. *In vitro* studies indicate that Nox4 knockdown is a promising strategy to reverse the adverse effects of hypoxia on actin polymerization in HBVSMC. Combined with recent reports that Nox4 inhibition promotes blood brain barrier stabilization and decreases hemorrhagic transformation after ischemic stroke [52, 53], our findings

strongly suggest that modulation of Nox4 production after cerebral ischemic injury could be used as a vasculoprotective strategy.

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Figure 1.

Hypoxia enhanced nitrotyrosine levels and actin depolymerization in HBVSMC. HBVSMCs were serum starved overnight, and then exposed to 30 min hypoxia/45 min reoxygenation in the presence of vehicle or selective nitration inhibitor epicatechin (Epi, 150μM, added during reoxygenation). HBVSMC were fixed and stained for F actin with Oregon Green 488-conjugated phalloidin fluorescent probe and for nitrotyrosine with an anti-nitrotyrosine primary antibody and an IgG conjugated to Cy5 secondary antibody. F actin and nitrotyrosine levels were detected by immunoblotting and immunostaining. (A) Hypoxia induced a shift in F:G actin in favor of G actin (n=4). (B) Hypoxia caused significant reduction in F actin levels compared to normoxia as shown by representative confocal microscopy images of HBVSMC stained for F actin cytoskeletal filaments (green) (n=3). (C) Hypoxia caused significant increase in nitrotyrosine levels compared to normoxia and Epi treatment nullified this effect (n=3–7). (D) Representative confocal microscopy images of HBVSMC stained for F actin cytoskeletal filaments (green) and colocalization of actin filaments with nitrotyrosine (red) are shown on top. Hypoxia enhanced nitrotyrosine levels and reduced F actin in HBVSMC compared to normoxia and inhibiting nitration with Epi restored F actin and nitrotyrosine levels back to normal (n=3–5) (*p<0.05, #p<0.01 vs normoxia, **p<0.01 and ***p<0.001 disease treatment interaction, post hoc Bonferroni analyses Ψp<0.05 vs vehicle normoxia or vehicle hypoxia).

Figure 2.

Hypoxia upregulated the expression of Nox4 and iNOS isoform in HBVSMC. HBVSMC were serum starved overnight, and then exposed to 30 min hypoxia/45 min reoxygenation. Nox4, Nox2, Nox1, iNOS and nNOS expressions were determined by western blot. Hypoxia upregulated Nox4 (A) and iNOS (D) expression compared to normoxic conditions. Hypoxia had no effect on Nox2 (B) Nox1 (C) and nNOS (E) expression in HBVSMC (* $p < 0.05$, *** $p < 0.001$ vs normoxia, $n = 4-6$).

Figure 3.

Silencing Nox4 expression in vitro

Nox4 expression was silenced in HBVSMC using electroporation-mediated siRNA delivery.

(A) Representative C_t values and the statistical analysis for the various groups. Hypoxia

increased Nox4 mRNA expression, and Nox4 silencing nullified this effect (n=3). (B)

Representative western blots of Nox4 and actin. Nox4 silencing reduced the expression of

Nox4 compared to scrambled under normoxic condition, and blunted the increase in Nox4

expression after hypoxia (n=3). (C,D) Representative western blots of Nox2 and Nox1.

Nox4 silencing had no effect on the expression of both Nox2 and Nox1 compared to

scrambled under normoxic conditions (n=3). (*p<0.01 disease treatment interaction)

Figure 4.

Nox4 silencing restored superoxide and nitrotyrosine levels in HBVSMCs after hypoxia. Nox4 expression was silenced in HBVSMC using electroporation-mediated siRNA delivery. Cells were switched to overnight serum-free media, and then exposed to 30 min hypoxia/45 min reoxygenation. HBVSMC were fixed and stained for F actin with Oregon Green 488-conjugated phalloidin fluorescent probe and for nitrotyrosine with an anti-nitrotyrosine primary antibody and an IgG conjugated to Cy5 secondary antibody. Nitrotyrosine levels were detected by immunoblotting and immunostaining. (A) Silencing Nox4 expression blunted the increase in nitrotyrosine levels after hypoxia compared to scrambled RNA (n=3–4). (B) Representative confocal microscopy images of HBVSMC stained for F actin cytoskeletal filaments (green) and colocalization of actin filaments with nitrotyrosine (red) are shown on top. Silencing Nox4 expression restored nitrotyrosine levels after hypoxia compared to scrambled RNA (n=3). (C) Effect of hypoxia and Nox4 silencing on superoxide formation were detected using fluorometric assay of dihydroethidium (DHE). Superoxide formation was significantly increased after hypoxia and Nox4 silencing levels reduced it back to normal (n=3–5). (D) Inhibiting iNOS with 1400W dihydrochloride abolished the increase in nitrotyrosine levels after hypoxia compared to the vehicle (n=5–9). (post hoc Bonferroni analyses *p<0.05 vs scRNA normoxia, Nox4 siRNA normoxia and hypoxia, **p<0.01 vs scRNA normoxia, ***p<0.001 vs scRNA hypoxia, p<0.05 vs scRNA normoxia and #p<0.01 vs scRNA hypoxia, **p<0.01 disease treatment interaction)

Figure 5.

Silencing Nox4 expression restored F actin levels in HBVSMCs after hypoxia. Nox4 expression was silenced in HBVSMC using electroporation-mediated siRNA delivery. Cells were switched to overnight serum-free media, and then exposed to 30 min hypoxia/45 min reoxygenation. F:G actin were determined by western blot. In another group, HBVSMC were fixed and stained for F actin with Oregon Green 488-conjugated phalloidin fluorescent probe. (A) Representative western blot of F and G actin is shown on top. Silencing Nox4 expression significantly increased F:G actin after hypoxia compared to cells treated with scrambled RNA (n=4). (B) Analysis of confocal microscopy images of F actin (green) showed that silencing Nox4 expression blunted the decrease of F actin after hypoxia (n=3). (post hoc Bonferroni analyses *p<0.05 vs scRNA hypoxia, p<0.05 vs scRNA normoxia).

Figure 6.

NADPH oxidase inhibition restored myogenic tone after I/R injury

Wistar rats were subjected to 30 min MCAO/45 min reperfusion. The NADPH oxidase inhibitor apocynin (50 mg/kg i.p) was administered at reperfusion. (A) I/R injury increased Nox4 expression in isolated MCAs compared to sham (n=3). (B) Nox2 expression was not altered by I/R injury (n=3). (C) I/R led to the loss of myogenic response in isolated MCAs (closed triangle) compared to sham (open triangles). Inhibiting NADPH oxidase nullified the effect of I/R injury and restored the myogenic response (closed square). MCAs isolated from sham rats treated with apocynin (open squares) experienced impaired myogenic response compared to sham (open triangles) (n=6–9). To detect role of Nox4, MCAs isolated from control Wistar rats were exposed to 30 min OGD/20 min reoxygenation with or without Nox4 inhibitor. (D) MCAs exposed to OGD experienced impaired myogenic reactivity (closed circle). Inhibiting Nox4 restored myogenic function (open circles) (n=3–8). (*p<0.001 disease treatment interaction, post hoc Bonferroni analyses **p<0.05 for sham vs sham+Apo, or ischemic vs sham and IS+Apo, ***p<0.001 vs sham, **p<0.01 vs sham and OGD+inh).